The Intracellular Free Amino Acid Pool Represents Tracer Precursor Enrichment for Calculation of Protein Synthesis in Cultured Fibroblasts and Myocytes[1]

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ABSTRACT We assessed the approach of using intracellular free amino acid enrichment as precursor enrichment for calculating the fractional synthetic rate of using the stable isotope tracer incorporation technique. We assumed that the true rate of protein synthesis was reflected by the rate of tracer incorporation over time divided by the plateau enrichment in protein. Isolated human fibroblasts and myocytes were cultured in medium supplemented with [^{15}N]glycine, [^{15}N]proline, and [d_5]phenylalanine. Culture medium and cells were collected daily from d 1 to 5. A portion of cells harvested on d 5 was subcultured for an additional 3 passages to d 20. Protein enrichments in both cell types reached a plateau after 20 d of cell culture. In fibroblasts, the true protein synthesis rates were 0.74, 0.85, and 0.86%/h, using protein plateau enrichments of [^{15}N]glycine, [^{15}N]proline, and [d_5]phenylalanine as precursor enrichments, respectively. When the corresponding intracellular free amino acid enrichments were used, protein synthesis rates were 0.76, 0.79, and 0.76%/h, respectively. Similarly, in myocytes, the true protein synthesis rates were 0.98 and 1.14%/h by protein plateau enrichments of [^{15}N]glycine and [d_5]phenylalanine, respectively. The synthesis rates were 0.94 and 1.01%/h by the corresponding intracellular enrichments, respectively. Extracellular amino acid enrichments resulted in underestimation of protein synthesis by a variable amount. We conclude that the intracellular free amino acid enrichment is an optimal surrogate for precursor enrichment to quantify protein synthesis. J. Nutr. 134: 1546–1550, 2004.

KEY WORDS: • *protein synthesis* • *stable isotopes* • *precursor enrichment* • *GC-MS* • *plateau enrichment*

Determination of protein synthesis using the tracer incorporation technique involves measuring the rate of incorporation of a tracer into the product over time and dividing by the enrichment of the precursor. In theory, the true precursor enrichment can be determined by continuing the tracer infusion until a plateau is reached in the product enrichment (1). Whereas this approach was used in vivo for specific proteins with a high turnover rate (2,3), it was not practical for determining the precursor enrichment in tissues with a slower turnover of protein, such as skin and muscle, because of the length of time necessary to reach a plateau in product enrichment. Consequently, in such circumstances it is necessary to use a surrogate to represent the true precursor enrichment.

Considerable research was directed toward assessing the most appropriate surrogate for the true precursor enrichment. Use of extracellular (i.e., plasma) amino acid enrichment generally results in a lower rate of protein synthesis than use of intracellular precursor enrichment because the extracellular enrichments are usually higher. However, it is not certain which, if either, is correct. Aminoacyl-tRNA enrichment has been used to better reflect the precursor for muscle protein synthesis, but accurate measurement is difficult and results have been variable (4–8). The so-called "flooding dose" technique (9) in which a large dose of the tracer + tracee molecules is given minimizes the uncertainty about precursor enrichment. However, the possible stimulation of protein synthesis by flooding dose is well recognized (10). Therefore, an optimum surrogate for precursor enrichment remains to be clarified.

In this study we assessed the approach of using intracellular amino acid enrichment as a surrogate for precursor enrichment in fibroblasts and myocytes. We used cell culture, a setting in which the true value for precursor enrichment can be identified. We focused on 2 cell types of physiological importance. Fibroblasts play a central role in skin protein metabolism and wound healing, and myocytes are a model for skeletal muscle. These 2 types of cells were cultured in medium supplemented with stable isotopes of [^{15}N]glycine, [^{15}N]proline, and [d_5]phenylalanine. Protein synthesis was calculated using intracellular and extracellular amino acid enrichments as precursors and compared to the values obtained when the plateau enrichments in protein-bound amino acid enrichments were used.

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[3] Abbreviations used: FSR, fractional synthetic rate; SA, specific activity; TTR, tracer to tracee ratio.

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METHODS

Cell culture. We used a human breast fibroblast cell line (CRL-1947, American Type Cell Culture). The cells were cultured in DMEM supplemented with 10% fetal bovine serum and 40 mg/L gentamycin sulfate. The cells were seeded at a density of 1×10^6 cells/dish and were incubated at 37°C in a humidified atmosphere of ambient air with 5% CO_2. The culture medium was changed every 48 h. Cells were grown for 6 passages to obtain a sufficient number of cells to perform the experiment. Starting at d 0, cells were grown in DMEM supplemented with stable isotope tracers (Cambridge Isotope Laboratories) of [^{15}N]glycine (0.68), [d_5]phenylalanine (0.31), and [^{15}N]proline (0.54). (Numbers in parentheses are mass ratios of labeled to unlabeled substrates.) This isotope-labeled culture medium was stored at 4°C and used throughout the entire experiment. Cell samples and culture medium were taken daily from d 0 to 5. Cells were detached from culture dishes with 0.25% trypsin in EDTA for 10 min at 37°C, followed by centrifugation (1500 × *g* for 10 min). Cell numbers were counted with a hemocytometer and viability was assessed by trypan blue. Cells reached confluence at d 5. At d 5, all cells in the culture dishes were harvested and a portion of cells was subcultured for an additional 3 passages (to d 20) using the same isotope tracer enriched culture medium. By then, most cells were newly synthesized. At each sampling time, cells from 20 dishes were pooled together and stored at −80°C for later analysis.

A human breast muscle cell line (CRC-2061, American Type Cell Culture) was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 40 mg/L gentamycin sulfate. Myocytes were cultured in the medium containing [^{15}N]glycine (0.91) and [d_5]phenylalanine (1.41). The culture experimental protocol was identical to that used with fibroblasts.

Analytical methods. Intracellular free amino acids and protein were isolated as previously described (11). Briefly, cells were homogenized 3 times at 4°C in 5% perchloric acid. The pooled supernatant was used to measure intracellular free amino acid enrichment. The pellet was washed and dried at 50°C overnight. The dried pellets were hydrolyzed in 6 mol/L constant boiling HCl. Amino acids from the cell supernatant and pellet hydrolysate were isolated using a cation exchange column (Dowex AG 50W-X8, Bio-Rad Laboratories) and dried in a rotary vacuum evaporator.

The isolated intracellular free amino acids and protein hydrolysates were derivatized with *N*-methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide at 100°C for 1 h for [d_5]phenylalanine and [^{15}N]glycine enrichment measurements. The enrichments of intracellular amino acids and protein-bound amino acids were measured by GC-MS. The abundance of ions was monitored at *m/z* 336 and 341 for phenylalanine and at *m/z* 160 and 161 for glycine. Intracellular free proline and protein-bound proline were measured using *N*-acetylester derivatization. The abundance of ions was monitored at *m/z* 200 and 201.

Amino acid concentrations in culture medium were measured by HPLC (Waters 1960, Waters).

Calculations. Fractional synthetic rates (FSR) for protein synthesis were calculated as

$$\mathrm{FSR} = \frac{E_{t2} - E_{t1}}{E_p \cdot (t_2 - t_1)},$$

where $E_{t2} - E_{t1}$ is the change in product enrichment (protein) between time points t_2 and t_1, and E_p is the mean precursor enrichment over the time ($t_2 - t_1$). The linear change of product enrichment (i.e., from d 1 to 3) was used to calculate the FSR. The true precursor enrichment for calculation of the FSR of protein is represented by the product plateau enrichment. For each tracer, the value of the FSR calculated with E_p equal to the product plateau enrichment was compared to the value when the corresponding enrichments of the intracellular free amino acids at plateau and the medium amino acid pools were used.

RESULTS

The concentrations of amino acids were constant in culture medium from d 1 to 5 in both fibroblasts and myocytes. The enrichments of amino acid tracers in culture medium were also constant from d 1 to 5.

Fibroblast culture

Cell growth. The mean cell numbers increased from d 0 at 1.2 million cells/dish to d 5 at 3.7 million cells/dish. There was a linear increase from d 0 to d 3, and the net FSR was 0.91%/h. By d 5, 33% of the cells were derived from initial unlabeled cells (old cells). After 3 subsequent passages, the percentage of old cells dropped to 1.2% at d 20. Because 98.8% of the cells at d 20 were newly synthesized cells from culture medium supplemented with labeled amino acids, it is reasonable to assume that isotopic equilibrium in the product was achieved at d 20. This assumption was supported by the data (see below).

Protein synthesis. The enrichments of [^{15}N]glycine (tracer to tracee ratio, TTR (m + 1)), [^{15}N]proline [TTR (m + 1)], and [d_5]phenylalanine [TTR (m + 5)] in culture medium were 0.675, 0.541, and 0.311, respectively. The intracellular enrichments of these tracers dropped to 0.292, 0.279, and 0.283, respectively. The changes of protein-bound [^{15}N]glycine [TTR (m + 1)], [d_5]phenylalanine [TTR (m + 5)], and [^{15}N]proline [TTR (m + 1)] were similar over time (**Fig. 1**). In all cases the protein-bound enrichment increased linearly for the first 3 d and essentially reached the plateau enrichment by d 5.

The FSR values were calculated using different precursors (**Table 1**). The values were similar with all 3 tracers (0.74, 0.85, and 0.86%/h for [^{15}N]glycine, [^{15}N]proline, and [d_5]phenylalanine, respectively). The intracellular free amino acid pool was a close representation of the product plateau enrichment for all amino acids, yielding FSR values with ±10% of the FSR values calculated with the product plateau value. The culture medium resulted in underestimating the FSR by a variable amount dependent on the specific amino acid tracer. The largest discrepancy between intracellular and extracellular enrichments (and therefore the biggest difference between calculated FSR values) was for glycine and the smallest difference was for phenylalanine.

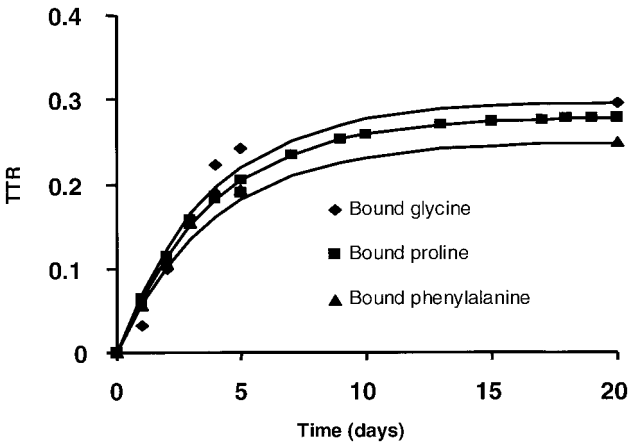

FIGURE 1 Changes of protein-bound amino acid enrichments in fibroblasts cultured in DMEM supplemented with stable isotope tracers of [^{15}N]glycine (0.68), [d_5]phenylalanine (0.31), and [^{15}N]proline (0.54). Numbers in parentheses are mass ratios of labeled to unlabeled substrates. Enrichments are expressed as TTR.

TABLE 1

Protein FSR calculated with different precursors in cultured fibroblasts[1]

Precursor	E_p (TTR)	$E_{t2} - E_{t1}$ (TTR)[2]	FSR (%/h)
[^{15}N]Glycine			
Product plateau	0.297	0.159	0.74
Intracellular free pool	0.292	0.159	0.76
Culture medium	0.675	0.159	0.33
[^{15}N]Proline			
Product plateau	0.257	0.158	0.85
Intracellular free pool	0.279	0.158	0.79
Culture medium	0.541	0.158	0.41
[d_5]Phenylalanine			
Product plateau	0.250	0.154	0.86
Intracellular free pool	0.283	0.154	0.76
Culture medium	0.311	0.154	0.69

[1] Data were collected from one study in fibroblasts cultured in DMEM supplemented with stable isotope tracers of [^{15}N]glycine (0.68), [d_5]phenylalanine (0.31), and [^{15}N]proline (0.54). Numbers in parentheses are mass ratios of labeled to unlabeled substrates. Enrichments are expressed as TTR. $E_{t2} - E_{t1}$ is the change in product enrichment (protein) between time points t_2 and t_1, and E_p is the mean precursor enrichment over the time ($t_2 - t_1$).

[2] $t_2 - t_1 = 72$ h.

Myocyte culture

Cell growth. The mean myocyte numbers increased from 0.8 million cells/dish at the outset to 2.5 million cells/dish after 5 d of cell culture. The fractional cell growth was 0.92%/h. By d 5, 68% of the cells were new cells grown from the isotope-labeled medium. At d 20, 99.0% of the cells were newly synthesized myocytes. Thus, the product enrichment at d 20 equaled the true precursor enrichment for calculation of protein FSR.

Protein synthesis. Only [^{15}N]glycine and [d_5]phenylalanine were used in culture medium to estimate myocyte FSR, because proline content is low in myocyte protein. Whereas the levels of protein bound tracer enrichments differed significantly between [^{15}N]glycine [TTR ($m + 1$)] and [d_5]phenylalanine [TTR ($m + 5$)], the pattern of change over time was similar (**Fig. 2**). Both tracers reached the plateau in protein-bound enrichment by d 5 (Fig. 2).

The tracer incorporation was rapid, so that the time between samples ($t_2 - t_1$) used for calculation of FSR was 48 h for [^{15}N]glycine and 24 h for [d_5]phenylalanine in order to ensure linearity of the increase in enrichment. The calculated rates of FSR from different potential precursors (E_p) ranged from 0.20 to 1.14%/h (**Table 2**). When the product plateau value was used, the FSR was similar, regardless of the tracer (0.98 and 1.14%/h for [^{15}N]glycine and [d_5]phenylalanine, respectively). In both cases, use of the intracellular free amino acid pool enrichment as E_p yielded similar values (0.94 and 1.01%/h for [^{15}N]glycine and [d_5]phenylalanine, respectively). However, there was a large discrepancy between those values and the FSR when the culture medium values of [^{15}N]glycine enrichment were used (0.20%/h), whereas use of the [d_5]phenylalanine culture medium enrichment yielded a value for protein FSR (0.99%/h) only slightly below the value obtained when the intracellular enrichment was used.

DISCUSSION

This study was designed to address the issue of the optimum precursor surrogate for precursor enrichment for the calculation of FSR. We assumed that the true rate of protein synthesis was reflected by the rate of tracer incorporation over time divided by the plateau enrichment in protein reached after 20 d of cell culture. In both myocytes and fibroblasts use of the intracellular free amino acid enrichment as the precursor resulted in a close approximation of the true rate of protein synthesis. Further, the different amino acid tracers yielded the same rates of protein synthesis despite widely varying contributions to protein content and differing gradients between the media and intracellular enrichments.

In protein synthesis, the charged tRNAs are assembled along the ribosome according to the codons of the mRNA. Measuring tRNA enrichment directly should therefore provide an optimal representation of the true precursor enrichment. However, accurate measurement of tRNA enrichment is very difficult. More tissue is required than is commonly available in human experiments, and the measurement is very tedious and requires great care and precision. A number of studies have compared measured values for tRNA enrichment

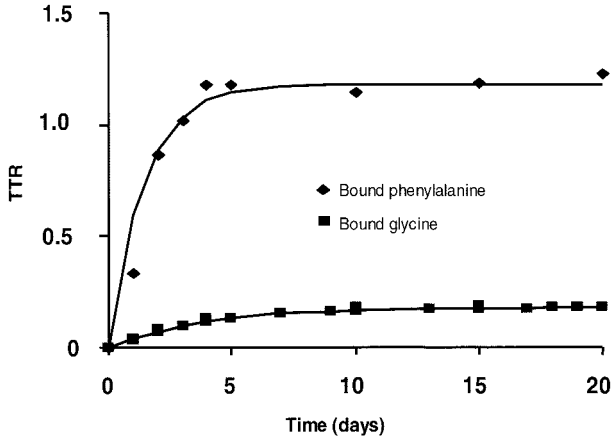

FIGURE 2 Changes of protein-bound amino acid enrichments in myocytes cultured in RPMI 1640 medium supplemented with stable isotope tracers of [^{15}N]glycine (0.91) and [d_5]phenylalanine (1.41). Numbers in parentheses are ratios of labeled to unlabeled substrates. Enrichments are expressed as TTR.

TABLE 2

Protein FSR calculated with different precursors in cultured myocytes[1]

Precursor	E_p (TTR)	$E_{t2} - E_{t1}$ (TTR)[2]	FSR (%/h)
[^{15}N]Glycine			
Product plateau	0.183	0.086	0.98
Intracellular free pool	0.191	0.086	0.94
Culture medium	0.907	0.086	0.20
[d_5]Phenylalanine			
Product plateau	1.22	0.334	1.14
Intracellular free pool	1.37	0.334	1.01
Culture medium	1.41	0.334	0.99

[1] Data were collected from one study in myocytes cultured in RPMI 1640 supplemented with stable isotope tracers of [^{15}N]glycine (0.91) and [d5]phenylalanine (1.41). Numbers in parentheses are mass ratios of labeled to unlabeled substrates. Enrichments are expressed as TTR. $E_{t2} - E_{t1}$ is the change in product enrichment (protein) between time points t_2 and t_1, and E_p is the mean precursor enrichment over the time ($t_2 - t_1$).

[2] $t_2 - t_1 = 48$ h for glycine and 24 h for phenylalanine.

with other possible surrogates, such as free intracellular amino acid enrichment, in hopes of validating a simpler approach than measuring tRNA enrichment. Unfortunately, these studies have yielded contradictions and therefore confusing results. For example, in 3 different studies the ratios of tRNA specific activity (SA) to the corresponding free extra- and intracellular SA were presented in heart cells studied in vitro (6–8). A ratio of 1.0 would thus mean that the surrogate marker (e.g., extracellular free amino acid) exactly reflected the tRNA enrichment. In 1 study (6) the extra- and intracellular phenylalanine enrichments were equal and essentially the same as tRNA (i.e., the ratios were close to 1). In another study (7), the extracellular SA was about twice the tRNA SA (i.e., tRNA SA/extracellular SA = 0.5), and the intracellular SA was less than the tRNA (tRNA SA/intracellular SA ≈ 1.3). In a third study (8), the relationship was found to be the reverse, in that the intracellular SA was higher than the tRNA SA, meaning that the ratio of tRNA SA/intracellular SA was less than 1. In this paper it is not clear how the intracellular enrichment could have actually exceeded the extracellular enrichment, because the tracer was added extracellularly. The picture is almost as confusing when muscle cells have been studied in vitro. One study found the intracellular SA to be lower than the tRNA and the extracellular SA to be greater than the tRNA SA (12). Similarly another found the tRNA SA to be considerably less than the extracellular SA (13). In contrast, a third study found the tRNA to be less than the intracellular enrichment (14). Clearly, these in vitro results do not clarify the relation between tRNA enrichment and either free intra- or extracellular amino acids. The discrepancies in results are likely explained by the difficulty in accurately determining the tRNA SA.

Studies have also been undertaken in vivo to address the same issue. A comprehensive study was performed by Bauman et al. (4) in swine given constant infusions of stable isotopes of both leucine and phenylalanine. They related both arterial and free muscle enrichment to the corresponding muscle tRNA enrichment. Both phenylalanine and leucine arterial enrichments exceeded the corresponding tRNA values by about 30%. The arterial α-ketoisocaproate enrichment, which should be a reflection of the pooled intracellular leucine enrichment from throughout the body, was closer to, but still greater than, the muscle tRNA enrichment. On the other hand, there was no significant difference between the tissue fluid phenylalanine enrichment and the corresponding tRNA enrichment. In contrast, the tissue fluid leucine enrichment exceeded the corresponding muscle tRNA enrichment. Taken together, the results of this study give reason to believe that the intracellular free amino acid enrichment is a reasonable surrogate for the true precursor for protein synthesis. However, if true, it is unclear why the leucine and phenylalanine tracers did not yield the same results, i.e., why the intracellular leucine did not equal the corresponding tRNA enrichment, whereas the intracellular phenylalanine enrichment did equal the tRNA enrichment. Phenylalanine and leucine are both taken up by the L and ASC transporters, and leucine but not phenylalanine is taken up by the A transporter as well. The A and ASC transporters are Na dependent, so they can generate a large concentration gradient, whereas the L transporter cannot. Given that leucine has a slightly larger concentration gradient than phenylalanine, the Na-dependent transporters may have a slightly greater effect on leucine. This could possibly contribute to differences in the distribution of extra- and intracellular enrichment, but our own data of measured values of intracellular and interstitial fluid enrichments indicate that the relation between extra- and intracellular enrichments of the 2 amino acids is comparable. Again, difficulty in precisely measuring tRNA enrichment accurately may have been a problem. This is suggested by the fact that in the only study performed in human volunteers in whom the free amino acid enrichment was compared with the tRNA enrichment, the leucyl tRNA and free intracellular leucine enrichments were found to be similar (15).

One approach to assessing the validity of the free intra- or extracellular enrichment as surrogate for precursor enrichment is to compare those values with the true precursor enrichment, reflected by the plateau in product enrichment. Cell culture provides an excellent system to prolong constant infusion and test this approach. This study demonstrated that the plateau product enrichments from 3 different amino acid tracers (at d 20 when >98.5% of cells are newly synthesized cells) were close to their intracellular free amino acid enrichments in both cell types. In contrast, there were major differences in the enrichments of the tracers in the extra- and intracellular spaces. In the myocytes, the extracellular glycine enrichment was 5-fold higher than the intracellular enrichment, whereas phenylalanine differed little between extra- and intracellular enrichment. In fibroblasts, the gradient between extra- and intracellular enrichment of glycine was less than in the myocytes, but still >100%. There was also a significant gradient from extra- to intracellular enrichment of proline that probably reflects de novo synthesis of the amino acid. Nonetheless, comparable values for FSR were attained with all tracers when intracellular amino acid enrichments were used.

There are 2 important points related to our findings. First, the discrepancy between enrichment in the extra- and intracellular free pools of glycine was likely due to the dilution of the intracellular pool by de novo synthesized glycine, which does not occur in the case of phenylalanine. Second, irrespective of differences in gradients between extra- and intracellular enrichment, the intracellular free enrichment accurately reflected the true precursor enrichment, regardless of the amino acid tracer. Thus, the precursor tracer for measurement of FSR does not need to be an essential amino acid (i.e., no de novo synthesis), provided that the intracellular free enrichment is measured.

Theoretically, the individual amino acid tracer used should not affect the rate of synthesis calculation, irrespective of the composition of the protein produced. This was true in our present experiment, despite a wide range in the relative contribution to the final product. Collagen is the predominant protein produced by fibroblasts. Collagen has a high abundance of both glycine and proline and a small proportion of phenylalanine. Nonetheless, all 3 tracers yielded the same protein FSR in this study. Because both glycine and proline can be synthesized within fibroblasts, their enrichment was markedly below the medium enrichment, yet the true precursor enrichment was accurately reflected by the intracellular enrichment. In vivo, the extracellular fluid compartment in skin is larger in relation to the intracellular fluid compartment than is the case in muscle. However, these compartments are commonly pooled when in vivo samples are measured. Thus, the tissue free amino acid pool, which combines interstitial and intracellular fluid, is likely to be more reflective of interstitial fluid enrichment in skin than in muscle. Further, the results of the current experiment indicate that the gradient from interstitial to intracellular enrichment for some amino acids, such as glycine, is larger for skin than it is for muscle. It is therefore not surprising that the tRNA enrichment in skin of individuals given [^{15}N]glycine was significantly lower than the tissue-free glycine enrichment (6). Thus, if the tissue-free glycine enrichment is used as the precursor for protein syn-

thesis, correction must be made for the contribution of the interstitial fluid glycine if an accurate estimate of precursor enrichment is to be obtained. Further, if a different amino acid, such as phenylalanine, is used as tracer that has a much smaller gradient from blood to intracellular enrichment, then a different rate of skin protein synthesis would likely be obtained if uncorrected tissue-free enrichment was used as the precursor for synthesis.

When these in vitro studies are interpreted in the context of the in vivo results, it is reasonable to conclude that the intracellular free amino acid pool generally reflects the true precursor for protein synthesis. However, it may be that leucine represents a specific exception to that conclusion. Although the discrepancy shown in Ref. (4) between intracellular and muscle tRNA enrichment when leucine is the tracer may simply reflect measurement inaccuracies, it is also possible that leucine has some unique properties. Thus, an in vitro study performed in cultured skeletal muscle cells by Schneible et al. (14) gives rise to the possibility of compartmentation of leucine for oxidation and for protein synthesis. Muscle cells were prelabeled with ^{14}C-leucine and then labeled medium was withdrawn and medium containing ^{3}H-leucine was added. With this approach it was possible to distinguish the percentage of precursor derived from the medium for oxidation and for synthesis (tRNA). The exact values were dependent on the leucine concentration in the medium, but at all concentrations tested most of the precursor for oxidation was derived from the medium, whereas most (but not all) of the leucine bound to tRNA was derived from the intracellular pool (14). Thus, it is possible that leucine may be compartmentalized within the cell. Even so, the results from the study of Schneible et al. (14) support the validity of the intracellular free leucine as a reasonable approximation of the true precursor enrichment for protein synthesis and are consistent with the paper showing correspondence between the muscle intracellular free leucine and leucyl-tRNA enrichment in human subjects (15).

Recycling of labeled amino acids from protein turnover and isotopic exchange from transamination reactions are potential problems in establishing isotopic steady state. However, the constant enrichments of protein bound amino acids during d 5 and 20 (Figs. 1 and 2) indicate either that the recycling and exchange effects were negligible or that they reached an equilibrium.

We performed the study once in each of the respective cell lines. Studies involving cell lines are typically not repeated because measurements are so reproducible. In this study, the plateau enrichments obtained at 10–20 d had a CV of 3% or less, compared to observed differences in enrichment as large as 80% between groups. Therefore, the differences in enrichment in the different pools reflect physiological differences rather than differences due to measurement error. The more pertinent question than statistical differences between precursors regards which value more closely reflects the true value. It is clear from Table 2 that in every case the intracellular free pool is reasonably close to the product plateau enrichment. In contrast, this is not the case for the culture medium enrichment. This leads to the conclusion that the intracellular free amino acid pool accurately reflects the true precursor enrichment for protein synthesis.

In summary, we validated intracellular free amino acid enrichment as surrogate for precursor enrichment for protein synthesis in cell culture. In both fibroblasts and myocytes, protein synthesis rates calculated from intracellular free amino acid enrichments were close to the true values of protein synthesis, irrespective of the tracer. Using extracellular amino acid enrichments resulted in underestimation of protein synthesis. This approach is applicable to in vivo studies in human subjects.

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